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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED OFFICE (DO/US)

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VACCINE DELIVERY SYSTEM AND METHOD OF PRODUCTION
Title of Invention

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Applicant(s) for DO/US

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To the United States Designated Office (DO/US):

I. Accompanying this transmittal letter are certain items which are required under 35 U.S.C. 371 in order that United States National processing of the above identified International application may commence:

(X) at the expiration of the applicable time limit under PCT Articles 22 and 39(1) according to the provisions of 35 U.S.C. 371(b).

( ) as soon as possible upon receipt of this express request under 35 U.S.C. 371(f).

1. The U.S. National fee [35 U.S.C. 371(c)(1)]

a. ( ) was previously transmitted by applicant on (date)____.

b. ( ) is submitted herewith as follows:

FOR	NO. FILED	NO. EXTRA	SMALL ENTITY RATE	FEE	or	OTHER THAN SMALL ENTITY RATE	FEE
Basic Fee	(USPTO NOT ISA OR IPEA)		////	$485	or	/////	**$970**
Total Claims	- 20 =	--	x 9 =		or	x18 =	
Ind. Claims	**2- 3**	--	x39 =		or	x78 =	
(X) Multiple Dependent Claim Presented			+130 =		or	+260 =	**260**
	TOTAL NATIONAL FEE		$____		or		$1230

i. ( ) A check in the amount of $_____ is enclosed.

ii. **(X)** Please charge the filing fee, multiple dependent claim fee (if applicable), excess independent claims fee (if applicable), and excess total claims fee (if applicable) to **Deposit Account No. 23-1703**.

iii. (X) The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-1703. A duplicate copy of this sheet is enclosed.

(iv) ( ) The filing fee is not enclosed.

2. A copy of the International application as filed [35 U.S.C. 371(c)(2)]:

a. **(X)** is transmitted herewith.

b. ( ) is not required as the application was filed with the United States Receiving Office.

c. ( ) has been transmitted

i. ( ) by the International Bureau. Date of mailing of the application (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed.

ii. ( ) by applicant on (date) _____.

3. A translation of the International application into the English language [35 U.S.C. 371(c)(2)]:

a. ( ) is transmitted herewith.

b. (X) is not required as the application was filed in English.

c. ( ) was previously transmitted by applicant on (date)_____.

4. Amendments to the claims of the International application under PCT Article 19 [35 U.S.C. 371(c)(3)]:

a. ( ) are transmitted herewith.

b. ( ) have been transmitted

i. ( ) by the International Bureau. Date of mailing of the amendments (from form PCT/IB/308): _____.

ii. ( ) by applicant on (date) _____.

c. (X) have not been transmitted as

i. ( ) no notification has been received that the International Searching Authority has received the Search Copy.

ii. ( ) the Search Copy was received by the International Searching Authority but the Search Report has not yet issued. Date of receipt of Search Copy (from form PCT/ISA/202): _____.

iii. ( ) applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210):

iv. (X) the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

5. A Translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]:

a. ( ) is transmitted herewith.

b. ( ) is not required as the amendments were made in the English language.

c. (X) has not been transmitted for reasons indicated at point I.4.b. or c. above.

6. A declaration for patent application of the inventor [35 U.S.C. 371(c)(4)] complying with 35 U.S.C. 115:

a. ( ) was previously submitted by applicant on (date) _____

b. **(X)** is submitted herewith; and such oath or declaration

i. **(X)** is attached to the application.

ii. **(X)** identifies the application and any amendments under PCT Article 19 which were transmitted as stated in points 1.2.b. or c. and 1.4. and states that they were reviewed by the inventor as required by 37 CFR 1.70.

c. ( ) will be submitted subsequently.

II. Concerning other documents:

1. An International Search Report or Declaration under PCT Article 17(2)(a):

a. ( ) has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed

b. ( ) is not required as the application was searched by the United States International Searching Authority.

c. ( ) A copy of the International Search Report is transmitted herewith.

d. ( ) has been submitted by applicant on (date) _____.

2. A Statement of prior art under 37 CFR 1.97 and 1.98:

   a. (**X**) is transmitted herewith including copies of the references cited on the attached form PTO-1449. Also included is a copy of the International-Type Search Report issued in the Swedish priority document.

   b. ( ) will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).

   c. ( ) was previously submitted by applicant on _____, in application serial no. _____.

3. (**X**) An Assignment is transmitted herewith for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

   a. (**X**) Please charge the $40 assignment recordation fee to Deposit Account No. 23-1703.

   b. ( ) A check in the amount of $___ is enclosed.

4. **Other document(s) or information included:**

   - Copy of the PCT/RO/101 - The PCT Request Form;
   - Six (6) sheets of formal drawings; and
   - Information Disclosure Statement, PTO-1449, copies of the references cited on the PTO-1449, and a copy of the International-Type Search Report (PCT/ISA/201/SE).

Respectfully submitted,

May 19, 1999
DATE

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enclosures

VACCINE DELIVERY SYSTEM AND METHOD OF PRODUCTION

FIELD OF THE INVENTION

The present invention concerns polymer particle vaccine delivery systems in which a water insoluble protein antigen is incorporated with particles comprising a polymer matrix. The present invention also concerns a method for incorporating such a water insoluble protein antigen with a polymer matrix in order to produce a polymer particle vaccine delivery system. In addition, the invention also provides a vaccine composition comprising the polymer particle delivery system. The vaccine can be used to treat and/or reduce the risk of for example *Helicobacter* infection.

BACKGROUND TO THE INVENTION

Several different types of vaccine delivery systems have been described in the literature (see e g "Vaccine Design. The subunit and adjuvant approach" (Eds: M F Powell and M J Newman), Pharmaceutical Biotechnology, vol 6, Plenum Press, NY 1995). Examples of known delivery systems for vaccines include liposomes, cochleates and polymer particles of a biodegradable or non-biodegradable nature. Antigens have also been associated with live attenuated bacteria, viruses or phages or with killed vectors of the same kind.

Polymer particles are well suited as vaccine delivery systems, because they can be produced in a range of sizes (eg, microparticles and nanoparticles) according, for example, to the preferred administration route for the vaccine and can slowly release the antigen inside the patient in order to build up a desirable immune response of the patient without the need for multiple vaccinations. The antigen is incorporated with the particles by encapsulation within a matrix of the polymer, with or without adsorption of the antigen onto the surface of the polymer particles

When the antigen is a protein, care must be taken to chose a preparation method for the polymer particles that does not remove the desired immunogenicity of the protein (eg, by denaturation). Thus, although various techniques are known for generally producing polymer particles with an active drug or substance, as explained below not all of these are well suited to use with a protein antigen.

The following general techniques have been used for preparing polymer particles:-

1. Hot Melt Microencapsulation (A.J. Schwope *et al Life Sci.* **1975**, *17*,1877);
2. Interfacial Polymerisation (G. Birrenbach & P. Speiser, *J. Pharm.Sci.* **1976**, *65*, 1763, Thies, In Encyclopaedia of Chemical Technology, 4 ed., Ed. Kirk-Othmer, **1996**, 16, p. 632);
3. Double Emulsion Solvent Evaporation Technique ("Vaccine Design. The subunit and adjuvant approach" (Eds: M F Powell and M J Newman), Pharmaceutical Biotechnology, vol 6, Plenum Press, NY 1995);
4. Double Emulsion Solvent Extraction Technique ("Vaccine Design. The subunit and adjuvant approach" (Eds: M F Powell and M J Newman), Pharmaceutical Biotechnology, vol 6, Plenum Press, NY 1995); and
5. Spray Drying (J. Cox, *et al.* WO 94/15636).

In the *Hot Melt Microencapsulation* method the matrix polymer is melted by heating while mixed with the active substance to incorporated with the particles. This technique is not well suited to use with a proteinaceous active substance, such as protein antigen, since the heating step tends to denature the active substance.

Interfacial Polymerisation is performed in following manner. A core material and the active substance are dissolved in a water immiscible solvent, together with a highly reactive monomer. This solution is then emulsified in water, where another monomer is dissolved, and a stable O/W emulsion is formed. An initiator is added to the water phase and polymerisation occurs, thereby forming a polymer particle incorporating the active substance. When the active substance is proteinaceous, the highly reactive monomer in the water immiscible solvent tends to react undesirably with the active substance as well as the

core material, which means that Interfacial Polymerisation is not well suited to the incorporation of a protein antigen with polymer particles.

Techniques are available which entail the formation of a water-in-oil (W/O) emulsion in which the active substance is dissolved in the W phase can be used to incorporate a proteinaceous active substance with polymer particles. Examples are the *Double Emulsion Solvent Extraction and Evaporation Techniques* and the *Spray Drying*.

For incorporation of a protein using the *Double Emulsion (W/O/X) Solvent Evaporation Technique*, a multiple W/O/X emulsion is used. The first step is the formation of a first (W/O) emulsion, in which the protein is dissolved in a first aqueous phase (W) and the oil (O) phase contains the matrix polymer and an organic solvent, the W and O phases being emulsified for example by ultra-sonication. In a second step, this first emulsion is then emulsified in a third phase (X) to form multiple W/O emulsion droplets dispersed in the X phase, which is commonly a second aqueous phase, but may for example be oil (eg sesame oil) instead. The organic solvent diffuses out from the droplets into the X phase before evaporating from the X phase. Thus, the organic solvent moves from the oil phase of the W/O emulsion droplets, to the X phase and then to the air. This results in a decrease of the organic solvent concentration in the O phase, and opposite an increase in the polymer concentration, since the polymer does not move with the organic solvent to the X phase. At a certain polymer concentration the polymer precipitates, thereby producing polymer particles comprising a matrix of the polymer incorporated with the protein (ie, protein is encapsulated within the matrix with or without surface adsorption onto the outside of the particle).

The *Double Emulsion (W/O/X) Solvent Extraction Technique* is similar to the Double Emulsion Solvent Evaporation Technique, but the organic solvent is extracted from the O phase of the W/O emulsion instead of being removed by evaporation. In addition, a second oil phase is used as the X phase in the double emulsion. The second oil phase extracts the organic solvent from the O phase, thereby raising the matrix polymer concentration in the O phase and leading to polymer particle formation in which the protein is incorporated

with the particles. (Lewis, *Drugs and the Pharmaceutical Sciences* (M Chasin and R Langer, eds.), Vol. 45, Dekker, New York, 1990, pp 1-42).

In the *Spray Drying Technique,* a W/O emulsion is formed as discussed above. The emulsion is sprayed through a nozzle to produce small droplets of the emulsion (dispersed in air) from which the solvent rapidly evaporates, thereby leading to formation of polymer particles incorporated with the protein. Microparticles in the 1-10 μm size range can be prepared (at relatively low cost) with this technique.

Biodegradable polymer particles are particularly well suited for use as vaccine delivery systems, because the polymer matrix itself is non-immunogenic and the encapsulation of the antigen protects it from degradation in the gastrointestinal tract (eg, by acid and proteases). An example of an especially suitable matrix polymer is PLG (poly(lactide-co-glycolide) copolymers - also known as PLGA and PLA). PLG particles have excellent tissue biocompatibility, biodegradability and regulatory approval. PLG particles degrade *in vivo* to form the non-toxic monomers, lactic- and glycolic acids and the release rate of incorporated active substances can be controlled by varying the molecular weight and copolymer ratio.

Examples of documents disclosing the use of Double Emulsion Techniques for incorporating *water soluble* proteins or peptides with PLG particles include:

H Rafati *et al*, "*Protein-loaded poly(DL-lactide-co-glycolide) microparticles for oral administration: formulation, structural and release characteristics*", J. Controlled Release **43** (1997), pp 89-102. This article discloses the use of a Double Emulsion (W1/O/W2) Solvent Evaporation Technique for incorporating bovine serum albumin (BSA) with particles of PLG.

M J Blanco-Príeto *et al*, "*Characterization and morphological analysis of a cholecystokinin derivative peptide-loaded poly(lactide-co-glycolide) microspheres prepared by a water-in-oil-in-water emulsion solvent evaporation method*", J. Controlled

Release **43** (1997), pp 81-87. This article discloses the incorporation of a small water soluble peptide with PLG particles. The authors observe that the stabilisation of the inner emulsion in the double emulsion by the combined use of OVA (ovalbumin) together with the use of a pH gradient between the inner and outer aqueous phase improved peptide encapsulation.

R V Diaz *et al*, "*Effect of surfactant agents on the release of ^{125}I-bovine calcitonin from PLGA microspheres: in vitro - in vivo study*", J. Controlled Release **43** (1997), pp 59-64. This article aims to investigate the possible influence that the surfactants Tween® -80 and Span® -60 (included in the W1 and O phases respectively) could have on the *in vitro* and *in vivo* release profile of ^{125}I-bovine calcitonin from PLGA microspheres. The article concludes that the protein encapsulation efficiency is similar independent of the presence or absence of the surfactants.

The prior art has therefore only concerned the incorporation of *water soluble* proteins and peptides with polymer particles using techniques which involve the formation of a W/O emulsion. The reason for this is that for the desired protein incorporation to take place, the protein must be solubilised in the W aqueous phase in order eventually to produce droplets of W/O emulsion in which the aqueous phase containing the solubilised protein provides the core of the droplets surrounded by the O phase which contains the matrix polymer in an organic solvent.

These techniques have not previously been considered to be useable for the incorporation of *water insoluble* proteins, because it was thought that these proteins cannot be suitably solubilised in the aqueous W phase.

Note that protein denaturation (eg, unfolding) by the organic solvent precludes the provision of the protein in an O phase together with the matrix polymer in order to produce polymer particles incorporated with a protein antigen.

WO 95/11009, WO 95/11010, WO 96/36317, US-A-5075109, US-A-4919929 and US-A-5529777 disclose the formation of microparticles incorporating water soluble antigens. None of these documents discloses the incorporation of a water insoluble protein antigen into polymer particles: WO 95/11009 and WO 95/11010 disclose the microencapsulation of MN rpg120 or QS21 into PLGA; WO 96/36317 discloses the formation of microparticles comprising a polymer matrix (eg, PLG) and a biological agent, further agents being optionally included in order to maintain the potency of the biological agent over the duration of the biological agent's release from the microparticles and to modify the release rate of the biological agent from the microparticles; US-A-5075109 discloses the formation of microparticles incorporating trinitrophenyl keyhole limpet hemocyanin or staphylococcal enterotoxin B as an antigen; and US-A-5529777 discloses the formation of microparticles by mixing a water soluble antigen with a solution of a water soluble polymer or hydrogel. US-A-5622649 discloses W/O and W/O/W emulsions, but there is no disclosure of forming polymer particles. It is essential for the invention disclosed in US-A-5622649 that no hydrophilic surfactant is present in the inner W phase. US-A-5622649 does not disclose a water insoluble protein antigen in the inner W phase.

SUMMARY OF THE INVENTION

We have now developed a method which does allow one to use techniques which involve the formation of a W/O emulsion, in order to produce a polymer particle vaccine delivery system in which a water *insoluble* protein antigen is incorporated with the particles.

Accordingly, the present invention provides a method for producing polymer particles for use as a vaccine delivery system in which a water insoluble protein antigen is incorporated with particles comprising a polymer matrix, wherein the method comprises:-

(a) mixing an aqueous phase (W) with an organic phase(O) that is immiscible with W to produce a W/O emulsion, in which the water insoluble protein is solubilised in the W

phase using a solubilising agent, and the O phase comprises the matrix polymer in an organic solvent;

(b) forming droplets of said W/O emulsion by dispersing the emulsion in a fluid medium, and removing said solvent from the O phase of the W/O emulsion droplets to thereby form polymer particles incorporating the water insoluble protein antigen; and

wherein in step (a) a stabilising agent is included in the W/O emulsion to promote the incorporation of the water insoluble protein with the polymer particles during step (b) by stabilising the W/O emulsion in the presence of said solubilising agent.

In addition, pursuant to the present invention we have for the first time provided a polymer particle vaccine delivery system in which a water *insoluble* protein antigen is incorporated with particles comprising a polymer matrix.

Furthermore, the present invention provides a vaccine composition comprising such a delivery system.

Another aspect of the present invention is the use of the delivery system in the manufacture of a vaccine composition, for the treatment of *Helicobacter* infection in a mammalian host, eg a human.

The present invention also relates to the use of the delivery system in the manufacture of a vaccine composition, for preventing or reducing the risk of *Helicobacter* infection in a mammalian host.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an SEM image of PLGA particles incorporated with HpaA according to the invention. The particles have an average diameter of 10μm.

Figure 2 is a particle size graph for PLGA particles produced according to the invention. The particles are incorporated with HpaA and have an average diameter of 10μm.

Figure 3 is an SEM image of PLGA particles incorporated with HpaA according to the invention. The particles have an average diameter of 300nm.

Figure 4 is a particle size graph for PLGA particles produced according to the invention. The particles are incorporated with HpaA and have an average diameter of 300nm.

Figure 5 shows the serum IgG2a results after intraduodenal immunisation with (i) HpaA co-administered with Cholera toxin (CT); or (ii) HpaA incorporated with PLG particles according to the invention. The bars represent the median of a group of six animals.

Legend to Figure 5 and abbreviations are as follows: units/ml= the mean of serum IgG2a levels of rats given three weekly i.p. immunisations with 100 μg HpaA and 25 μg Cholera toxin was arbitrarily set to 50 units/ml; HpaA+CT=100 μg HpaA admixed with 25 μg Cholera toxin (n=4), HpaA/PLG=PLG formulation containing 100 μg HpaA (n=6).

Figure 6 shows the mucosal IgA results after intraduodenal immunisation with (i) HpaA co-administered with Cholera toxin (CT); or (ii) HpaA incorporated with PLG particles according to the invention. The bars represent the median of a group of six animals. "OD405" refers to the optical density read-out of anti-HpaA ELISA.

Legend to Figure 6 and abbreviations are as follows: OD405=Optical density read-out of anti-HpaA ELISA; HpaA+CT=100 μg HpaA admixed with 25 μg Cholera toxin (n=4), HpaA/L121=PLG formulation containing 100 μg HpaA (n=6).

DETAILED DESCRIPTION OF THE INVENTION

As explained above, the present invention provides a polymer particle vaccine delivery system and a process for its production, in which a water insoluble protein is incorporated with particles comprising a polymer matrix. The polymer particles can be in the form of microparticles, such as microspheres.

The term "protein" in "water insoluble protein" is defined as a protein, polypeptide or peptide. The present invention contemplates the use of one or more proteins, so that the vaccine carrier system that is produced in some embodiments comprises more than one protein incorporated with the polymer matrix. In this case, at least one of the proteins is a water insoluble protein antigen; each of the other proteins can be water soluble or insoluble, and may or may not be antigenic (eg it may act as an adjuvant to promote the antigenicity of the water insoluble protein).

Water insoluble proteins are distinguished from water soluble proteins by their ability to associate with non-ionic detergents to form micelles, whereas water soluble proteins do not associate with such detergents to form micelles (A Practical Guide to Enzymology, C H Suelter, John Wiley & Sons Publishers, ISBN 0-471-86431-5, pp 71-72). Water insoluble protein/non-ionic detergent micelles can be easily detected, because the electrophoretic mobility of the protein when incorporated in the micelles is different from the protein's electrophoretic mobility in the absence of the non-ionic detergent. Since water soluble proteins do not associate with non-ionic detergents, there is no change in electrophoretic mobility for these proteins when in the presence or absence of a non-ionic detergent. The first step is to mix the protein to be tested with a non-ionic detergent, then with an ionic detergent. If micelles are formed (ie the protein is water insoluble), the ionic detergent subsequently added becomes incorporated in the micelles thus changing the electrophoretic mobility of the protein.

Another test for water insoluble proteins is as follows (A Practical Guide to Enzymology, C H Suelter, John Wiley & Sons Publishers, ISBN 0-471-86431-5, pp 71-72). The protein

is dispersed in Triton®X-114 at 0°C. When the temperature of this detergent is raised above 20°C, its cloud point, separation into two phases occurs: an aqueous phase and a detergent phase. Water soluble proteins are recovered in the aqueous phase, whereas water insoluble proteins are found in the detergent phase.

In the method of the present invention, a W/O emulsion is formed in which the water insoluble protein is solubilised by the solubilising agent in the aqueous W phase, and the matrix polymer is dissolved in the O phase along with the organic solvent. Formation of the W/O emulsion can, for example, be effected by mixing the W phase containing the solubilised protein with the O phase containing the dissolved matrix polymer. The mixture is then emulsified, eg by ultra-sonication, stirring, extrusion, high shear mixing or high pressure homogenisation, while one or more suitable stabilising agents are included in the mixture. In this respect, (i) one or more stabilising agents can be included in the W phase , but not in the O phase, prior to mixing; or (ii) one or more stabilising agents can be included in each of the W and O phases prior to mixing; or (iii) one or more stabilising agents can be included in the O phase, but not in the W phase, prior to mixing. For forming the W/O emulsion, the W phase is mixed with the O phase in a ratio by volume of less than 1, more preferably 1:10,000 to 1:1, even more preferably, 1:100 to 1:1. The most preferred range is 1:4 to 1:1.5 for particularly good protein antigen incorporation.

In a second step, the stabilised W/O emulsion is dispersed in a fluid medium (ie, a liquid medium or gaseous medium such as air) to remove the organic solvent. This raises the concentration of the matrix polymer in the O phase so that the droplets "harden" and thereby form polymer particles incorporated with the water insoluble protein.

The second step can be carried out in various ways. In one embodiment, the method of the present invention can form part of a Double Emulsion (W/O/X) Solvent Evaporation Technique in which in the second step, the stabilised W/O emulsion is dispersed in a further liquid phase (X) which is immiscible with the O phase to produce a W/O/X double emulsion comprising stabilised W/O droplets from which the solvent is evaporated,

thereby producing the polymer particles incorporating the water insoluble protein antigen. Dispersal of the stabilised W/O emulsion in the X phase can be carried out for example by ultra-sonication. The X phase should be immiscible with the O phase or have only low (limited) miscibility with the O phase. Suitable examples for the X phase include aqueous phases, triglyceride (eg, sesame oil) and silicone oil.

In another embodiment, the method of the present invention is a Double Emulsion (W/O/X) Solvent Extraction Technique in which in the second step, a W/O/X double emulsion comprising W/O droplets is produced in a similar way to the Double Emulsion Solvent Evaporation Technique. In the Solvent Extraction embodiment, however, the X phase extracts the solvent from the O phase of the droplets, thereby producing the polymer particles incorporating the water insoluble protein antigen. As with the Solvent Evaporation Technique, suitable X phases for the Extraction Technique should have no or low (limited) miscibility with the O phase, and examples include an aqueous phase, triglyceride (eg, sesame oil) and silicone oil. In the Solvent Extraction Technique, the volume ratio of the X phase to the O phase is, however, considerably larger than with the Evaporation Technique.

If desired for these double emulsion techniques, one or more stabilising agents can be included in the X phase. The stabilising agents used for stabilising the first (W/O) emulsion can be used for this purpose. Optionally, removal of the solvent can be accelerated by stirring the double emulsion and/or warming (not to a protein antigen denaturing temperature) the double emulsion and/or reducing the pressure inside a vessel containing the double emulsion.

In yet another embodiment, the method of the present invention is a spray drying technique in which in the second step the stabilised W/O emulsion is dispersed in a gaseous medium (eg air) to form a spray of stabilised W/O emulsion droplets from which the solvent evaporates, thereby producing the polymer particles incorporating the water insoluble protein antigen. The W/O emulsion is usually dispersed by pumping it through a nozzle

having a fine aperture. Spraying into a warmed chamber (ie not at protein denaturing temperature) can be effected in order to promote solvent evaporation.

Another embodiment involves the use of a fluid gas technique in the second step for forming the polymer particles. These techniques involve supercritical fluid technology. A supercritical fluid is a fluid simultaneously at or above its critical pressure and critical temperature. An example of a suitable fluid gas technique for the present embodiment is Gas Anti-Solvent Precipitation (GAS). In the conventional GAS technique, a substance of interest is dissolved in a solvent and a supercritical fluid (eg, carbon dioxide) is introduced into (mixed with) the solution, leading to the rapid expansion of the volume of the solution. As a result, the solvating power of the solvent decreases dramatically over a short period of time, thereby triggering the precipitation of particles (Cf. J W Tom and P G Debendetti, J Aerosol. Sci, 22 (1991), 555-584; P G Debendetti *et al*, J Controlled Release, 24 (1993), 238-257; EP 437451 and EP 322687). When applied to the present invention, the stabilised W/O emulsion is used in place of the solution and a fluid gas is introduced with the solution to lead to a rapid expansion of the stabilised W/O emulsion, and thus formation of polymer particles incorporated the water insoluble protein antigen.

A modification of the GAS technique is the SEDS (Solution Enhanced Dispersion By Supercritical Fluid) technique (WO 95/01221 and WO 96/00610), and this can be used in the second step of the method of the invention for forming polymer particles. Here, one can use material in its supercritical or near supercritical state, or compressed gas as the "fluid gas". The supercritical fluid can be, for example, selected from carbon dioxide, nitrous oxide, sulphur hexafluoride, xenon, ethylene, chlorotrifluoromethane, ethane and trifluoromethane.

Other suitable techniques (modified anti-solvent (GAS) techniques) sinclude: Precipitation with Compressed Anti-Solvents (PCA) procedure (Dixon *et al., AIChE Journal,* **1993**, *39*, 127-139; and Supercritical Anti-Solvent (SAS) procedure (Yeo *et al., Biotech.Bioeng.*,**1993**, *41*, 341-346) or ASES (DE744329).

When fluid gas techniques have been used in the prior art, proteins have been included directly in an organic phase containing, eg ethanol (see EP-0542314; Tom *et al., In Supercritical Fluid Engineering Science, ACS Symposium Series,* **1993**, *514*, 238-257) or DMSO (see WO 96/29998), for co-precipitation with polymer. Disadvantages are the low solubility of proteins in organic solvents and supercritical fluids/modified supercritical flows (Stahl *et al*, "Dense Gas Results", *Fluid Phase Equilibria,* **1983**, *10*, 269); and protein denaturation (eg unfolding) by the organic solvent (Dill, K.A and Shortle, D. *Ann. Rev. Biochem.* **1991**, *60*, 795-825).

None of the prior art discloses the use of a W/O emulsion together with an anti-solvent fluid gas technique like GAS, SEDS, ASES, SAS and PCA, as in the preferred embodiment of the present invention. This avoids the disadvantages of the prior art techniques in which the protein is included directly in an organic phase, while providing an efficient method for producing polymer particles incorporated with a water insoluble protein, for use as a vaccine delivery system.

In the general method of the present invention, the water insoluble protein must be solubilised in the W phase, and for this a solubilising agent such as a hydrophilic surfactant or chaotropic agent can be used. More than one solubilising agent may optionally be used, ie one or more hydrophilic surfactant, one or more chaotropic agent, or one or more hydrophilic surfactant together with one or more chaotropic agent. The solubilising agent is hydrophilic, and by the term "hydrophilic surfactant" is meant a surfactant that solubilises a water insoluble protein antigen and is itself soluble in water (ie in the W phase); surfactants that are soluble in the O phase, but not in the W phase are not included in the term "hydrophilic surfactant". Preferably, the hydrophilic surfactant has a hydrophile-lipophile balance (HLB) of 10 or more; most preferably 13 or more. It will be readily apparent to the skilled person how to determine HLB values. In addition, reference is made to the following publications, which concern the determination of HLB values: Griffin, W. C., *J. Soc. Cosmet. Chem.* **1949**, *1*, 311; Griffin, W. C., *J. Soc. Cosmet. Chem.*

1954, *5*, 249; Davies, J. T., *Proc. 2nd Int. Cong. Surf. Acitivity*; London, 1957, p. 417; Davies, J. T.; Rideal, E. K., *Interfacial Phenomena*; Academic: New York-San Fransisco-London, 1963. p 129; Davies, J. T., *Progress in Surface Science*; Danielli, J. F., Parkhurst, K. G. A., Riddford, A. C., Eds.; Academic: New York, 1964; Vol. 2, p 129.

Suitable temperatures at which the W/O emulsion is formed are from 0°C to the boiling point on the O phase, but excluding temperatures that would denature the protein in the emulsion. Room temperature is often a suitable working temperature, although it should be mentioned that lower temperatures are preferable to slow down the dynamics of the emulsion.

Suitable hydrophilic surfactants include one or a mixture of surfactants selected from non-ionic, anionic, cationic and zwitterionic surfactants.

Suitable non-ionic surfactants can be selected from alkyl-glucopyranosides(eg, decyl-, dodecyl-, or octyl-glucopyranoside), alkyl-thioglucopyranosides (eg, octyl-thioglucopyranoside), alkyl-maltosides (eg, dodecyl- or lauryl maltoside), alkoyl-methyl glucamides (eg, heptanoyl-, octanoyl-, nonanoyl-, or decanoyl-N-methyl glucamide), polyoxyethylene alcohols (eg, $C_{11}E_8$, LuBrol PX or Brij series), polyoxyethylene alkyl phenols (eg, polyoxyethylene octyl phenols such as Nonidet P-40, Triton X-100), emulphogens, polyoxyethylene sorbitol esters (eg, Tween series), polyoxyethylene fatty acid esters, hydrophilic polyoxyethylene alkyl ethers and digitonin.

Suitable anionic surfactants can be selected from cholates (eg, sodium salts of glyco- or taurocholate), alkylsulphonates (eg, the sodium salt of pentyl-, octyl-, decyl-, dodecyl-, or myristylsulphonate), deoxycholates (eg, sodium deoxycholate), alkyl sulphates (eg, the sodium salt of octyl-, decyl-, dodecyl- or myristylsulphate), oligooxyethylene dodecyl ether sulphates and sodium dodecylsarcosinate.

Suitable cationic surfactants can be selected from alkylpyridinium salts (eg, a bromide or chloride of cetyl-, myristyl-, dodecyl- or decylpyridinium) and alkyltrimethylammonium salts (eg, cetyl-, myristyl-, dodecyl- or decyl-trimethylammonium bromide or chloride).

Suitable zwitterionic surfactants can be selected from CHAPS (3-[(3-cholamidopropyl dimethylammonio]-1-propanesulphonate), CHAPSO (3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate), BIGCHAP (N, N-*bis*[3-D-Gluconamidopropyl]-cholamide), deoxy BIGCHAP (N, N-*bis*[3-D-Gluconamidopropyl]-deoxycholamide), lyso phosphatidylcholine (eg, C16 lyso PC), N-tetradecyl-N,N-dimethyl-3-ammonia-1-propane sulphonate, alkylbetaines (eg, dodecylbetaine) and sulphobetaines.

Where a hydrophilic surfactant is used as a solubilising agent, a suitable range for the surfactant is to provide the surfactant in the W phase (in this case, we mean a solution including all of the components of the W phase except the protein(s) to be solubilised) at a concentration of 0.1 to 100 times, preferably 0.1 to 10 times, and more preferably 0.1 to 5 times the Critical Micelle Concentration (CMC) of the surfactant. Thus, the surfactant could for example be used at its CMC. Where a mixture of hydrophilic surfactants is used to solubilise, these ranges relate to the CMC of the mixture.

Suitable chaotropic agents include one or more of a perchlorate, thiocyanate, guanidine, chlorate, iodide, bromide, nitrate and urea.

While a hydrophilic solubilising agent is necessary to achieve solubilisation of the water insoluble protein in the aqueous (W) phase, the required hydrophilic character has the undesirable effect of destabilising any W/O emulsion droplets formed, by favouring a phase inversion to an O/W emulsion (ie, the aqueous phase being the outer continuous phase). According to the present invention, we include a stabilising agent in the W/O emulsion specifically to counter the undesirable effect of the solubilising agent (while retaining the agent's solubilising capacity) and exert a dominating effect which favours, and therefore stabilises, the W/O emulsion while forming the W/O droplets and forming

the polymer particles. In this way, the stabilising agent brings about (promotes) the incorporation of the water insoluble protein with the polymer particles.

The common feature of all stabilising agents is that they adsorb to the W/O interface in the emulsion to prevent or reduce coalescence of the W droplets emulsified in the O phase. The stabilising agent may be soluble in the W phase and/or the O phase. More than one stabilising agent may optionally be included in the W/O emulsion.

Suitable stabilising agents that are soluble in the O phase, for example, increase the viscosity of the O phase of the W/O emulsion and/or are surface active agents having a predominantly hydrophobic character (hydrophobic surfactants). By the term "hydrophobic surfactant" is meant a stabilising surfactant that is overall predominantly hydrophobic and is soluble in the O phase, but not in the W phase.

Preferably, the stabilising agent is used in a proportion of from 0.01 to 99% by weight of the W/O emulsion , more preferably from 0.02 to 50 or 25% by weight of the W/O emulsion, and most preferably, from 0.05 to 5% by weight of the W/O emulsion. A particularly preferred embodiment is the use of 0.3% by weight of the W/O emulsion.

Preferably one or more stabilising agent is used, each agent being selected from polymers, polar lipids, and hydrophobic surfactants.

A preferred stabilising polymer is selected from poly(vinyl pyrrolidone), poly(vinyl alcohol), polysaccharides, polyethyleneoxide and water soluble proteins (eg, gelatin; bovine serum albumin).

A preferred polar lipid is selected from cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, glycolipids and phosphatidic acid.

A stabilising agent can be used that is a non-ionic, hydrophobic surfactant selected from a sorbitan fatty acid ester (SPAN™ series), hydrophobic polyoxyethylene alkyl ether, sucrose ester, alkyl-glucopyranoside, polyglycerol polyricinoleate and block-copolymers of ethylene oxide with propyleneoxide and/or lactic acid.

A stabilising agent can be used that is an anionic, hydrophobic surfactant selected from an alkylsulphate salt, dialkylsulphosuccinate salt, alkylbenzene sulphonate salt and a fatty acid salt. Particularly preferable examples include sodium 1,4-bis (2-ethylhexyl) sulphosuccinate, calcium dioleate and aluminium or zinc stearate.

A stabilising agent can be used that is a cationic, hydrophobic surfactant selected from an alkyltrimethylammonium salt and a dialkyldimethylammonium salt (eg, distearyl dimethylammonium bromide).

In one preferred embodiment, a suitable stabilising composition is polyoxyethylene sorbitan fatty acid ester mixed with a sorbitan fatty acid ester. In another preferred embodiment, poly(vinyl pyrrolidone) and sodium 1,4-bis (2-ethylhexyl) sulphosuccinate are used together as stabilising agents.

In one embodiment of the present invention, the matrix polymer can be used as a stabilising agent by increasing the viscosity of the O phase. In this case, the matrix polymer can be added to the O phase up to the saturation point of the matrix polymer, with or without the use of another stabilising agent.

A suitable matrix polymer can be a homo- or co-polymer selected from polyesters, polyanhydrides, polyorthoesters, polycarbonates, polyamides, poly(amino acids), polyacetals, polycyanoacrylates, polyacrylates, biodegradable polyurethanes, non-erodable polyurethanes, polymers of ethylene-vinyl acetate, acyl substituted cellulose acetates, polysaccharides, polystyrenes, polyvinyl chloride, polyvinyl fluoride, poly(vinyl imidazole), chlorosulphonated polyolefins, polyethylene oxide, polyethers and

polyoxalates. Optionally, mixtures of two or more of these polymers can be used in the O phase as matrix polymers.

In one embodiment, the polymer is an poly(esteramide).

Preferred matrix polymers are polyester homopolymers, such as polylactic acid, polyglycolic acid, polyhydroxybutyrate, poly(alpha hydroxyacids) and polycaprolactone.

Other preferred matrix polymers are polyester co-polymers, such as poly(lactide-co-glycolide), poly(lactic-co-glycolic acid), poly(hydroxybutyrate-hydroxyvalerate) and poly(lactide-co-caprolactone).

A particularly preferred matrix polymer is poly(D,L-lactide-co-glycolide).

In order to dissolve the matrix polymer in the O phase, one or more organic solvent may be used. Where we make reference to removing the organic solvent from the O phase in the method of the present invention, this should be construed as removing the organic solvent mixture, where more than one solvent is used. Suitable organic solvents depend on the particular matrix polymer used, and the skilled person can readily determine which solvent to use in order to dissolve the matrix polymer. Noteable examples of suitable solvents include methylene chloride, chloroform and ethyl acetate.

The method of the present invention can be used to produce polymer particles with an average diameter ranging from 0.01 to 1000μm according to the volume size distribution. For subcutaneously implanted vaccine delivery systems, an average diameter from 0.1 to 100μm according to the volume size distribution is preferred.

For mucosal delivery *in vivo*, an average diameter from 0.05 to 20μm according to the volume size distribution is preferred, with a range of 0.1 to 10μm according to the volume size distribution being the most suitable. Examples of suitable mammalian mucosa include the buccal, nasal, tonsillar, gastric, intestinal (small and/or large intestine), rectal and

vaginal mucosa. Appropriate administration routes for these vaccines include oral, nasal, rectal and vaginal administration, with the oral, nasal and rectal routes being most preferred.

It will be readily apparent to the skilled person how to use the method of the present invention to produce particles of a desired size range. This skilled person could routinely, for example vary the relative volume ratios of the W and O phases and/or vary the thoroughness of emulsion homogenisation (eg, by varying the speed and/or duration of emulsion stirring).

When the delivery system of the present invention is provided as part of a vaccine composition, it can optionally be so provided in combination with a suitable adjuvant. Suitable adjuvants are Cholera toxin (CT), *E. coli* heat labile toxin, cytokines and chemokines. The vaccine compositions can be used to treat and/or prevent a diseased state or infection (depending on the antigen(s) of the delivery system) in a mammalian patient by administering an immunologically effective amount of the composition to the patient. The term "immunologically effective amount" means an amount which elicits an immune response by the patient to the antigen(s) carried by the delivery system. An "immune response" is a response which eradicates, suppresses, prevents and/or reduces the risk of the infection or disease in the patient. Typically, an appropriate dose of the or each antigen per administration would be approximately 10μg to 10mg, preferably approximately 50μg to 5mg, for oral administration. Suitable dosage forms include a frozen dispersion, freeze-dried particles or a liquid dispersion.

Examples of Preferred Embodiments of The Present Invention

The present invention can be used to provide a polymer particle delivery system that can be included in a vaccine composition for the treatment and/or prophylaxis of *Helicobacter* (in particular *Helicobacter pylori*) infection in a mammalian host. Thus such a vaccine can be administered to a patient, eg orally, in order to effect the treatment and/or prophylaxis. By

"treatment", we mean the eradication or suppression of an existing *Helicobacter* infection in the host (in this respect, general reference is made to WO 96/40893). By "prophylaxis", we mean preventing or reducing the risk of the mammal becoming infected by *Helicobacter* after the vaccine has been administered.

The gram-negative bacterium *Helicobacter pylori* is an important human pathogen, involved in several gastroduodenal diseases. Colonisation of gastric epithelium by the bacterium leads to active inflammation and progressive chronic gastritis, with a greatly enhanced risk of progression to peptic ulcer disease.

In order to colonise the gastric mucosa, *H. pylori* uses a number of virulence factors. Such virulence factors comprise several adhesins, with which the bacterium associates with the mucus and/or binds to epithelial cells; ureases which helps to neutralise the acid environment; and proteolytic enzymes which makes the mucus more fluid.

Despite a strong apparent host immune response to *H. pylori*, with production of both local (mucosal) as well as systemic antibodies, the pathogen persists in the gastric mucosa, normally for the life of the host. The reason for this is probably that the spontaneously induced immune-response is inadequate or directed towards the wrong epitopes of the antigens.

In order to understand the pathogenesis and immunology of *H. pylori* infections, it is of great importance to define the antigenic structure of this bacterium. In particular, there is a need for characterisation of surface-exposed (like adhesins) and secreted proteins which, in many bacterial pathogens, have been shown to constitute the main virulence factors, and which can be useful for the diagnosis of *H. Pylori* and in the manufacture of vaccine compositions. Monoclonal antibodies (MAbs) against membrane preparations of *H. pylori* have been disclosed by Bölin *et al.* (1995) J. Clin. Microbiol. 33, 381-384. One of these MAbs, designated HP30-1:1:6, reacted with a 30 kDa protein which was shown to be exposed on the surface of intact bacteria and to have properties like that of an adhesin.

When the method of the present invention is used during the production of a vaccine for the treatment and/or prophylaxis of *Helicobacter* infection, the water insoluble protein antigen is a *Helicobacter* protein or antigenic fragment thereof. Preferably, the protein antigen is a *Helicobacter pylori* protein or antigenic fragment thereof (eg, a membrane-associated or membrane-bound antigen). A *Helicobacter* protein that is a protein expressed on the outer surface of *Helicobacter* provides a particularly good antigen for incorporation with the polymer matrix of the vaccine delivery system.

Whenever stressed or threatened, the *H. pylori* cell transforms from a bacillary to a coccoid form. In the coccoid form, the *H. pylori* cell is much less sensitive to antibiotics and other anti-bacterial agents. Circumstantial evidence indicate the *H. pylori* might be transmitted between individuals in this form, possibly via water or direct contact. An efficient vaccine composition should therefore elicit an immune response towards both the coccoid and the bacillary form of *H. pylori*. Preferred water insoluble *Helicobacter* proteins for the vaccine delivery system are therefore such proteins that are exposed on the surface of both the dividing (bacillary) and resting (coccoid) forms of *Helicobacter*.

Since systemic immunity probably only plays a limited role in protection against mucosal *Helicobacter* infection, it is also important that the vaccine composition will enhance protective immune mechanisms locally in the stomach.

Reference is made to WO 96/38475 which discloses an antigen that is a putative adhesin and is exposed on the surface of both the dividing (bacillary) and resting (coccoid) forms of *Helicobacter pylori*. The disclosure of WO 96/38475 is hereby incorporated by reference, and in particular the expression methods disclosed therein are expressly incorporated by reference and the skilled person is directed to these specific disclosures for further guidance. We refer to this antigen as a HpaA protein. Cloning of a *hpaA sequence*, which reportedly coded for a 20 kDa receptor-binding subunit of the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin (NLBH) of *H. pylori*, has been disclosed by Evans *et al.*

(1993) J. Bacteriol. 175, 674-683. Reference is also made to P W Toole *et al*, Bacteriology Vol. 177, No. 21, Nov. 1995; and Jones,A.C., Logan,R.P., Foynes,S., Cockayne,A., Wren,B.W. and Penn,C.W., J. Bacteriol. 179 (17), 5643-5647 (1997) which concern HpaA proteins.

The Hpa A protein is expressed by all *H. pylori* strains tested , and antibodies created towards this protein do not cross-react with common endogenous human bacteria of other species or with selected human tissues including the gastric mucosa. Thus being a well conserved putative adhesin with immunogenic properties, the HpaA protein is useful both for the detection of *H. pylori* infections as well as for the manufacture of vaccine compositions. Table 1 shows a comparison of HpaA amino acid sequences derived from 4 different strains of *H. Pylori*. It can be seen from the table that the sequence is highly conserved amongst different strains.

Table 1

```
Evans (8826)    MKTNGHFKDFAWKKCLLGTSVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
GTC (J99)       MKTNGHFKDFAWKKCFLGASVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
Trust (17874)   MKTNGHFKDFAWKKCLLGASVGALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
Penn (11637)    MRANNHFKDFAWKKCLLGASVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
TIGR (26695)    MKANNHFKDFAWKKCLLGASVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
                *::*.**********:**:** ************************************

Evans (8826)    LKPAFQYSDNIAKEYENKFKNQTTLKVEEILQNQGYKVINVDSSDKDDFSFAQKKEGYLA
GTC (J99)       LRPAFQYSDNIAKEYENKFKNQTTLKVEEILQNQGYKVINVDSSDKDDFSFAQKKEGYLA
Trust (17874)   LRPAFQYSDNIAKEYENKFKNQTVLKVEQILQNQGYKVINVDSSDKDDFSFAQKKEGYLA
Penn (11637)    LRPAFQYSDNIAKEYENKFKNQTALKVEQILQNQGYKVISVDSSDKDDFSFAQKKEGYLA
TIGR (26695)    LRPAFQYSDNIAKEYENKFKNQTALKVEQILQNQGYKVISVDSSDKDDLSFSQKKEGYLA
                *:**********************.****:**********.********:**:********
```

```
Evans (8826)     VAMIGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFVKVTILEPMSGESLDSF

GTC (J99)        VAMNGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFVKVTILEPMSGESLDSF

Trust (17874)    VAMNGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFVKVTILEPMSGESLDSF

Penn (11637)     VAMNGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFIKVTILEPMSGESLDSF
```

```
TIGR (26695)    VAMNGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFVKVTILEPMSGESLDSF
                *** ************************************:****************


Evans (8826)    TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFASIMQEMDKKLT
GTC (J99)       TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFASIMQEMDKKLT
Trust (17874)   TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFGSIMQEIDKKLT
Penn (11637)    TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFANIMQEIDKKLT
TIGR (26695)    TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFANIMQEIDKKLT
                ************************************************..****:*****


Evans (8826)    QRNLESYQKDAKELKNKRNR
GTC (J99)       QRNLESYQKDAKELKNKRNR
Trust (17874)   QKNLESYQKDAKELKGKRNR
Penn (11637)    QKNLESYQKDAKELKGKRNR
TIGR (26695)    QKNLESYQKDAKELKGKRNR
                *:*************.****
```

"*" at a certain position denotes an identical amino acid in all sequences

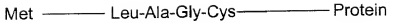
"." at a certain position denotes conserved amino acids (eg, amino acids of the same charge type such as lysine or arginine at a certain position).

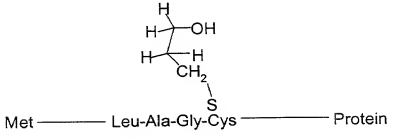
Penn (11637)	DNA sequence deposited in Genbank under Accession No. X92502
Trust (17874)	DNA sequence deposited in Genbank under Accession No.U35455
Evans (8826)	DNA sequence deposited in Genbank under Accession No.X61574
TIGR (26695)	DNA sequence deposited under Accession No. AE000591
GTC (J99)	DNA sequence obtained in-house.

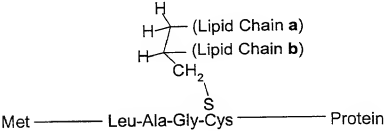
The strain names are indicated in brackets, strain 8826 being obtained from SWISS-PROT accession Q48264.

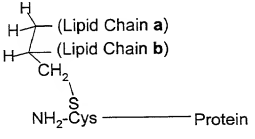
The accompanying sequence listing shows a nucleic acid sequence (SEQ ID NO. 1) comprising the *hpaA* gene and the amino acid sequence (SEQ ID NO. 2) of a HpaA protein that is predicted to be 29 kDa, which includes a signal sequence and is encoded by the *hpaA* gene. Note that in SEQ ID NO's. 1 and 2, amino acid 222 is serine; this position can alternatively be arginine (see SEQ ID NO's. 3 and 4).

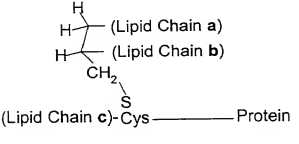
Referring to the formulae below, the predicted 29 kDa protein is shown as formula **I**. We believe that this is further processed in *Helicobacter* as follows. Protein **I** is processed by the enzyme prolipoprotein glyceryltransferase to give a product **II**, followed by the addition of two lipid chains to give product **III**. The latter step is catalysed by at least one transacylase. The signal sequence of the lipidated product **III** is then cleaved off by prolipoprotein signal peptidase to give a product **IV** (it is believed that the signal sequence corresponds to positions 1 to 27 in SEQ ID NO's. 2 and 4). A third lipid chain is then added to this protein by phospholipid diglyceride lipoproteintransacylase to give a "fully lipidated" protein product (**V**). In the method of the present invention, a lipidated form of HpaA can be used as the water insoluble protein. Most preferably, a fully lipidated form of HpaA is used (ie, one with at least 3 lipid chains, eg three C16 chains as in protein **V**). The protein part (referred to here as the "protein core") of product **V**, therefore, corresponds to the full length protein (**I**) minus the signal sequence. It is contemplated that the protein core (or even antigenic fragments thereof) can be synthesised *in vitro* and lipidated (not necessarily in the same pattern as product **V**), and this lipidated HpaA protein can be used as a water insoluble protein antigen in the present invention.

I 
Met
Leu-Ala-Gly-Cys
Protein

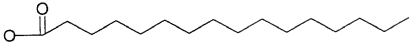
II 
H
H
OH
H
H
CH2
S
Met
Leu-Ala-Gly-Cys
Protein

III 
H
H
(Lipid Chain a)
H
(Lipid Chain b)
CH2
S
Met
Leu-Ala-Gly-Cys
Protein

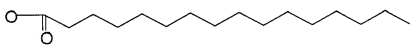
IV 
H
H
(Lipid Chain a)
H
(Lipid Chain b)
CH2
S
NH2-Cys
Protein

V 
H
H
(Lipid Chain a)
H
(Lipid Chain b)
CH2
S
(Lipid Chain c)-Cys
Protein

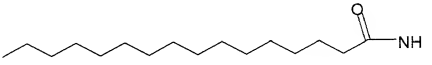
Where "(Lipid Chain **a)**" denotes:-



"(Lipid Chain **b)**" denotes:-



"(Lipid Chain **c)**" denotes:-



Preferably, where a lipidated HpaA protein antigen is used as a water insoluble protein in the present invention, the protein part of the antigen has an amino acid sequence that is identical or substantially similar to positions 28 to 260 of the amino acid sequence set out in SEQ ID NO.2 or 4, but of course retaining antigenic activity suitable for use in a vaccine for prophylaxis and/or treatment of *Helicobacter* infection in a mammalian host (including the ability of the antigen to elicit a mucosal as well as a systemic immune response against *Helicobacter* in a mammalian host). By "substantially similar" we mean one or more of the following: the protein part includes an amino acid sequence at least 60%, 70%, 80%, 90%, 95%, 98% or 99% homologous to positions 28 to 260 of the amino acid sequence set out in SEQ ID NO.2 or 4; the protein part includes at least 5, 10, 20, 50, 100, 150 or 200 contiguous amino acid residues of positions 28 to 260 of the amino acid sequence set out in SEQ ID NO. 2 or 4, but retaining antigenic activity (ie at least one immunogenic epitope, with or without fusion to an inert or immunologically active carrier polypeptide) suitable for use in a vaccine for prophylaxis and/or treatment of *Helicobacter* infection in a mammalian host; the protein part of the antigen includes an amino acid sequence differing in amino acid sequence by 1, 2, 3, 5, or 10 residues from positions 28 to 260 of the amino acid sequence set out in SEQ ID NO. 2 or 4, but retaining antigenic activity suitable for use in a vaccine for prophylaxis and/or treatment of *Helicobacter* infection in a mammalian host.

Specific mention is made to the section in WO 96/38475 describing how to identify and analyse epitopes of the HpaA protein.

The protein antigen used in the invention may be prepared from *Helicobacter* cells and/or produced by recombinant techniques.

Although in the formulae above, a fully lipidated HpaA protein is shown where each of the three lipid chains has 16 carbons, each lipid chain can be a C12 to C20 lipid chain. C16 and C18 lipid chains are preferred, and most preferably, the HpaA antigen has at least one C16 chain and at least one C18 chain. It is known that lipid modification can determine immunological properties of bacterial lipoproteins (see Weis, J J *et al* (1994) Infection and

Immunity, vol. 62, 4632-4636). Where the protein has three C16 lipid chains (protein V above), the protein has a predicted weight of 27 kDa. The weight of HpaA protein may, however, vary depending on the lipidation pattern of the protein.

PREPARATION OF POLYMER PARTICLES INCORPORATED WITH HpaA

The following examples illustrate the incorporation of HpaA with either PLGA particles or PHB (poly(3-hydroxybutyrate)) particles.

Analysis of Emulsions

The visual appearance of the double emulsions was studied with light microscopy (Leica microscope, DMRBE, Leica Mikroskopie und Systeme GmbH, Germany).

Analysis of Particles

The particle size, form and morphology were studied with scanning electron microscopy. Degree of agglomeration and particle size distribution were analysed with an Aerosizer (Aerosizer®, Amherst Process Instruments, Hadley, MA, USA). This measurement technique is based on the determination of the aerodynamic time-of-flight for the particles. The density of the particles assumed to be equal to the polymers density, 1.25 g/cm^3 for PHB.

Particle sizes were also determined by means of laser diffraction, using a Coulter LS130 (Coulter Corp, Hialeah, Florida, USA).

Determination of HpaA Loading

PHB particles

a) Total protein content:

Particles (3-10 mg) were dissolved in 300 µl chloroform. SDS-laemmli (400 µl) was then added and the protein was extracted from the organic phase to the water phase. The samples were shaken at 60°C for 30 min. The water phase was heated to 95°C for 15 min and the protein content analysed by polyacrylamide gel electrophoresis (SDS-PAGE). The SDS Laemmli reagent solution used in the protein analysis consisted of 1.25 ml TRIS HCl 2 M (pH 6.8) buffer solution, 5.05 g glycerol (99%), 0.8 g sodium dodecylsulphate (SDS), 1 ml 2-mercaptoethanol, 1 µl bromophenol blue and 10 ml water.

PLGA particles

a) Total protein content:

Particles (3-10 mg) were dissolved in 1 ml acetone. The protein precipitate was centrifuged for 15 minutes at 17 530xg, and about 2/3 of the supernatant was removed with a Hamilton syringe. Pure acetone was added in order to wash the sample twice. The remaining acetone was evaporated by vacuum centrifugation. SDS-Laemmli (200 µl) was added and the sample was heated to 95°C for 15 minutes. The analysis of the protein content was performed by SDS-PAGE.

b) Analysis of the amount of the surface associated protein:

Analysis of the amount of protein associated to the surface was performed according to Rafati et al. (*Journal of Controlled Release* **1997** *43*, 89-102). To 5-6 mg of particles was added 2 ml 2 % (w/v) SDS in water. The samples were shaken for 4 hours. The samples were then centrifuged at 2700xg for 3 minutes and the water phase removed to a new tube. The water was evaporated by vacuum centrifugation and 1 ml Laemmli (without SDS) was added. The water phase was heated to 95°C for 15 min and the protein amount analysed by SDS-PAGE.

A: Double Emulsion Techniques

In the following examples, the 27kDa lipidated form of HpaA (ie protein V above) was used. The HpaA polypeptide antigen was obtained in-house.

Example 1

PLGA particles incorporated with the HpaA protein were produced to an average diameter of approximately 10μm according to the volume size distribution , which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), PVP (poly(vinyl pyrrolidone), Mw 10 000, Aldrich), acetone, NOG (n-Octyl-glucopyranoside, SIGMA), TRIS buffer salts and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 950 μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was mixed with 1050 μl 2% (w/w) PVP (aq). The solution was dispersed in 3900 μl 3% (w/w) PLGA (DCM) by homogenization at 20000 rpm for 3 min. The formed W_1/O emulsion was further dispersed in 140 g 10 % (w/w) PVA (aq) by homogenization at 5 000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate. The particles were collected by centrifugation and washed with water to remove the PVA.

The volume average diameter of the particles was determined to 9.4 μm by laser diffraction measurements. The degree of protein antigen incorporation with the PLGA particles was determined by SDS-PAGE to be 49 % of the protein that was initially added.

After protein content analysis, the antigen concentration in the suspension was adjusted to 0.33 g/l.

Results: Figure 1 shows an SEM image for particles of Example 1. Figure 2 shows the particle size distribution of particles of Example 1.

Example 2

PLGA particles incorporated with the HpaA protein were produced to an average diameter of approximately 300nm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™ 502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23000, Aldrich), PVP (poly(vinyl pyrrolidone), Mw 10 000, Aldrich), AOT (sodium 1,4-bis (2-ethylhexyl) sulphosuccinate, SIGMA), acetone, NOG (n-Octyl-glucopyranoside, SIGMA), TRIS buffer salts and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 950μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was mixed with 1050 μl 2% (w/w) PVP (aq). The solution was dispersed in 3900 μl of a DCM solution, containing 3% (w/w) PLGA and 0.4% (w/w) AOT, by homogenization at 20 000 rpm for 3 min. The formed W_1/O emulsion was further dispersed in 140 g 10 % (w/w) PVA (aq) by homogenization at 5 000 rpm for 5 min. The formation of double

emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate. The particles were collected by centrifugation, washed with water to remove the PVA and freeze dried.

The volume average size diameter of the particles was determined to 0.35 μm/1.7 μm (bimodal distribution) by laser diffraction measurements. The HpaA content (% of dry particles) was calculated to 0.3% (w/w).

Results: Figure 3 shows an SEM image for particles of Example 2. Figure 4 shows the particle size distribution of particles of Example 2.

Example 3

PLGA particles incorporated with the HpaA protein were produced to an average diameter of approximately 300nm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), PVP (poly(vinyl pyrrolidone), Mw 10 000, Aldrich) and AOT (sodium 1,4-bis (2-ethylhexyl) sulphosuccinate, SIGMA) were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 500 μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was mixed with 500 μl 2% (w/w) PVP (aq). The solution was dispersed in 1950 μl of a DCM solution, containing 3% (w/w) PLGA and 0.4% (w/w) AOT, by homogenization at 20 000 rpm for 3 min. The formed W_1/O emulsion was further dispersed in 70 g 10 %

(w/w) PVA (aq) or 70 g 2 % (w/w) PVA by homogenization at 5 000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate. The particles were washed with water by cross flow filtration.

The volume average size of the particles was determined to 0.35 μm/1.7μm (bimodal distribution, 10 % (w/w) PVA) and 0.29μm (2% (w/w) PVA) by laser diffraction measurements.

Example 4

PLGA particles incorporated with the HpaA protein were produced to an average diameter of 6μm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), PVP (poly(vinyl pyrrolidone), Mw 10 000, Aldrich). The water was of ELGA quality (18.2 MΩ).

Methods: 500 μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was mixed with 100 μl 10% (w/w) PVP (aq). The solution was dispersed in 1950 μl 3% (w/w) PLGA (DCM) by homogenization at 20 000 rpm for 3 min. The formed W_1/O emulsion was further dispersed in 70g 10 % (w/w) PVA (aq) by homogenization at 5 000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate.

The volume average size of the particles was determined to 6μm by laser diffraction measurements.

Example 5

PLGA particles incorporated with the HpaA protein were produced to an average diameter of approximately 6μm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™ 502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), PVP (poly(vinyl pyrrolidone), Mw 10 000, Aldrich) and AOT (sodium 1,4-bis (2-ethylhexyl) sulphosuccinate, SIGMA) were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 500 μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was mixed with 100 μl 10% (w/w) PVP (aq). The solution was dispersed in 1900 μl of a DCM solution, containing 3% (w/w) PLGA and 0.26% (w/w) AOT, by homogenization at 20 000 rpm for 3 min. The formed W_1/O emulsion was further dispersed in 70 g 10 % (w/w) PVA (aq) by homogenization at 5 000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate.

The volume average size of the particles was determined to 5.8 μm by laser diffraction measurements.

Example 6

PLGA particles incorporated with the HpaA protein were produced to an average diameter of 4μm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™ 502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), Span 85 (ICI) and Tween 80 (Merck-Schuchardt) were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 400 μl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was dispersed in 1500 μl of a DCM solution, containing 3% (w/w) PLGA and 0.4% (w/w) Span™ 85/Tween™ 80 (ratio: 80/20 by weight), by probe sonication at 65 W output for 5 min. The formed W_1/O emulsion was further dispersed in 56 g 10 % (w/w) PVA (aq) by homogenization at 5 000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate.

The volume average size of the particles was determined to 4μm by laser diffraction measurements.

Example 7

PLGA particles incorporated with the HpaA protein were produced to an average diameter of 150nm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactide-co-glycolide, 50:50, Mw 14400, RESOMER™ 502, Boehringer Ingelheim), DCM (dichloromethane), PVA (poly(vinyl alcohol), Mw 13-23 000, Aldrich), Tween™ 80 (Merck), Span™ 85 (Speciality Chemicals) and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 200 µl of a 2% (w/w) NOG solution (10mM, pH8 TRIS buffer), with the antigen, was dispersed in 800 µl of a DCM solution, containing 3% (w/w) PLGA and 0.4% (w/w)) Span™ 85/Tween™ 80 (ratio: 80/20 by weight), by probe sonication at 65 W output for 10 min. The formed W_1/O emulsion was further dispersed in 10ml 10 % (w/w) PVA (aq) by sonication at 65 w for 10 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight to allow the DCM to evaporate. The particles were collected by centrifugation and washed with water to remove the PVA.

The volume average size of the particles was determined to 130 nm/480 nm by laser diffraction measurements. The degree of protein antigen incorporation with the PLGA particles was determined by SDS-PAGE to be 44 % of the protein that was initially added.

Example 8

PLGA particles incorporated with the HpaA protein were produced to an average diameter of approximately 13 µm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactic-co-glycolic acid, 50:50, Mw 14400, RESOMER™ 502, Boehringer Ingelheim), DCM (dichloromethane), PGPR (polyglycerol polyricinoleate, Danisco), PVA (poly(vinyl alcohol), Mw 13-23000, Aldrich), NOG (n-Octyl-glucopyranoside, SIGMA), TRIS buffer salts and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 950 µl of an aqueous solution (10 mM TRIS buffer, pH8) containing 2 % (w/w) NOG and the antigen was dispersed in 1.74 g DCM solution containing 1.8 % (w/w) PGPR and 10 % PLGA (w/w) by high-shear mixing at 20000 rpm for 3 min. The so-obtained W_1/O emulsion was further dispersed in 50 g 10 % (w/w) PVA (aq.) by high-shear mixing at 6000 rpm for 5 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight in an open beaker to allow the DCM to evaporate. The particles were collected by centrifugation and washed with water to remove the PVA.

The average diameter of the particles was determined to 12.6 µm by laser diffraction measurements. The protein antigen content in the dry PLGA particles was determined by SDS-PAGE to be 0.2 % (w/w), which corresponds to an encapsulation degree of 44 % of the protein that was initially added.

Example 9

PLGA particles incorporated with the HpaA protein were produced to an average diameter of 9 µm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PL(D,L)GA (poly D,L-lactic-co-glycolic acid, 50:50, Mw 6000, RESOMER™ 502 H, Boehringer Ingelheim), DCM (dichloromethane), PVP (poly(vinyl pyrrolidone) Mw 10 000, Aldrich), PVA (poly(vinyl alcohol), Mw 13-23000, Aldrich), NOG (n-Octyl-glucopyranoside, SIGMA), TRIS buffer salts and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 700 µl of an aqueous solution (10 mM TRIS buffer, pH8) containing 2 % (w/w) NOG and the antigen was mixed with 1200 µl 2 % (w/w) PVP (aq). This solution was dispersed in 3900 µl DCM solution containing 3 % PLGA (w/w) by high-shear mixing at 20000 rpm for 3 min. The so-obtained W_1/O emulsion was further dispersed in 141 g 10 % (w/w) PVA (aq.) by high-shear mixing at 5000 rpm for 6 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight in an open beaker to allow the DCM to evaporate. The particles were collected by centrifugation and washed with water to remove the PVA.

The volume average diameter of the particles was determined to 9.3 µm by laser diffraction measurements. The protein antigen content in the dry PLGA particles was determined by SDS-PAGE to be 0.4 % (w/w), which corresponds to an encapsulation degree of 93 % of the protein that was initially added.

Example 10

PHB particles incorporated with the HpaA protein were produced to an average diameter of 3 μm according to the volume size distribution, which is well suited to gastric mucosal delivery.

Materials & Methods

Materials: PHB (poly(3-hydroxybutyrate), Mw 63 500, Astra Tech), DCM (dichloromethane), PVP (poly(vinyl pyrrolidone) Mw 10 000, Aldrich), PVA (poly(vinyl alcohol), Mw 13-23000, Aldrich), NOG (n-Octyl-glucopyranoside, SIGMA), TRIS buffer salts and Laemmli sample buffer were used as purchased. The water was of ELGA quality (18.2 MΩ).

Methods: 950 μl of an aqueous solution (10 mM TRIS buffer, pH8) containing 2 % (w/w) NOG and the antigen was mixed with 1050 μl 2 % (w/w) PVP (aq). This solution was dispersed in 3900 μl DCM solution containing 3 % PHB (w/w) by high-shear mixing at 20000 rpm for 3 min. The so-obtained W_1/O emulsion was further dispersed in 141 g 10 % (w/w) PVA (aq.) by high-shear mixing at 6000 rpm for 3 min. The formation of double emulsion droplets was confirmed by light microscopy. The $(W_1/O)/W_2$ double emulsion was stirred overnight in an open beaker to allow the DCM to evaporate. The particles were collected by centrifugation and washed with water to remove the PVA.

The volume average diameter of the particles was determined to 3.2 µm by laser diffraction measurements. The protein antigen content in the PHB particle suspension was determined by SDS-PAGE. The overall protein yield was determined to 34 %.

B: Fluid Gas Techniques

Example 11

Poly(3-hydroxybutyrate) (PHB) particles incorporated with the HpaA protein were produced.

General Technique

Particles were prepared in a SEDS apparatus (Bradford Particle Design, Bradford, UK) from a stabilised W/O emulsion containing the water insoluble protein antigen.

The emulsion and the anti-solvent (CO_2) were introduced in a coaxial nozzle, which is located inside a pressure vessel which is was located in an oven. Under controlled pressure and temperature conditions, the anti-solvent extracts the organic solvent from O phase of the formed emulsion droplets. The concentration of the matrix polymer in the droplets is thereby increased, leading to rapid particle formation. The particles were collected in a vessel, while the anti-solvent and the extracted organic solvent emerged through a back pressure regulator.

The nozzle used was a three component nozzle connected, either in a sandwich mode or in a two-solutions mode, with an opening of 0.2 mm in diameter. In the sandwich mode, the supercritical fluid passes through the innermost and the outermost passage, while the emulsion passes through the intermediate passage. In the two solution mode, the emulsion

and a modifier, eg ethanol, are mixed just before contact with the fluid gas. (The modifier increases the solubility of water in the fluid gas in order to enhance water extraction.) The fluid gas passes through the outer passage, the modifier through the intermediate passage and the emulsion through the inner passage.

Materials & Methods

Materials: Poly(3-hydroxybutyrate) (PHB, Astra Tech, Sweden, molecular weight (MW) 63 500 g/mol), n-Octyl-β-D-glucopyranoside (NOG), poly(vinylpyrrolidone) (PVP, Aldrich, Germany, MW 10 000 g/mol), AOT (sodium 1,4-bis (2-ethylhexyl) sulphosuccinate, SIGMA. Methylene chloride (99.5 %) was used as organic solvent and carbon dioxide as a supercritical fluid. Ethanol (99.5%) was used as a modifier in supercritical processing.

Method: PHB was dissolved in methylene chloride at 2 bar, 90°C. Equal volumes of 2% (w/w) PVP (aq) and HpaA stock solution [1.11 mg/ml HpaA in TRIS-HCl buffer (10 mM, pH 8) and 2% (w/w) NOG], were mixed. This mixture (3.8 ml) was injected (during homogenisation at 20000 rpm) to 15.2 ml methylene chloride containing 1% (w/w) PHB and 0.4% (w/w) AOT in a 25 ml Kinematica dispersion vessel. The total homogenisation time was 3 minutes. The homogeniser used was a Polytron PT3100, Rotor PT-DA 3012/2 (Kinematica AG, Switzerland). All procedures were performed under ambient conditions.

Two runs were made with this stabilised W/O emulsion with different running conditions in the SEDS apparatus. The run MPP63 was done by using a three-component nozzle in the two solution mode by using ethanol (flow rate 0.5 ml/min) as a modifier. In MPP64 the sandwich mode was used (Table 2).

Table 2. SEDS processing of emulsion

Batch	Modifier	P (bar)	T (°C)	Flow rate CO_2 (ml/min)	Flow rate emulsion (ml/min)
MPP63	ethanol	180	50	26	0.1
MPP64	-	240	35	26	0.1

According to SEM graphs, the particle size was 1-3 μm for both trials (MPP63 and MPP64).

Theoretical composition of particles should be 55.8% (w/w) PHB, 43.5% (w/w) surfactants and 0.6% (w/w) HpaA. The analysis of the amount of HpaA gave a result of 0.4% HpaA of the total weight of the particles for both MPP63 and MPP64.

In vivo Testing of Vaccine Delivery Systems According To The Invention

An *in vivo* rat model was used for screening of antibody levels in response to duodenally administered antigen delivery systems according to the preferred embodiment of the invention. This model is based on the use of Spraque-Dawley rats equipped with a chronic duodenal fistula for administration of delivery systems. IgG2a and IgA levels are investigated in blood and mucus samples respectively.

Material and Methods:

A. Operation of rats: Insertion of a chronic duodenal fistula.

A fistula made of Plexiglas is inserted into the duodenum to enable administration of drugs intraduodenaly.

Anaesthesia: Ketamin (Ketalar® 50 mg/ml) + Xylazin (Rompun® 20 mg/ml) mixed 8+1. There was given 0,2 ml/kg body weight ip.

Procedure: Shave and disinfect the abdomen. Make a 2-3 cm long midline incision. Make a pouch-suture with a non absorbable material (e.g. Ticron 5-0 or 4-0) about 0.5-1 cm from the pylorus in the duodenum. Make a hole in the duodenum with a 18Gx1" cannula and widen it with a small pair of tweezers. Put the cannula in the hole and tighten the suture so that it fits in with the narrow part of the fistula. Flush the fistula with physiological saline to make sure that it does not leak and to clear the fistula from blood. Make an incision laterodorsally 2 cm from the midline on the right-hand side about 1.5 cm caudal the last rib. Let the incision go through the skin and the abdominal wall. Loosen the skin around the incision. Put the fistula through the incision and suture the "wings" from the fistula to the abdominal wall with a non absorbable material (e.g. Ticron 5-0 or 4-0). Suture the midline with a absorbable material (e.g. Dexon 4-0) and the skin with non absorbable material (e.g. Dermalon 4-0). Put the cap on the fistula. Give about 10 ml fluids (e.g. Rehydrex with Glucose 25 mg/ml) subcutaneously.

B. Administration of Delivery Systems.

Upon administration the rats are put into Bollman-cages (a procedure for which rats are trained during a week following operation and prior to administration). The cap from the fistula is unscrewed and replaced with a catheter. A delivery system is administered with a syringe and a blunt cannula which exactly fits with the catheter. Close the catheter with forceps, withdraw the first syringe and put a new syringe with physiological saline onto the catheter, open the forceps and flush the rest content of the drug into the duodenum. Close the catheter with the forceps again and withdraw the syringe. Leave the catheter closed for about 5 minutes before the catheter is removed and the cap is put on the fistula.

C. Sampling of blood and mucus.

Measurements of antibody production were made following duodenal administration of antigen, delivery system including antigen, antigen + adjuvant or delivery system (including antigen) + adjuvant. The rats are administered once weekly and are terminated 10 weeks after the last immunisation. Upon termination samples are taken and evaluated. Every two weeks after the last immunisation blood-samples are collected and evaluated.

D. Antibody analysis

Specific antibodies against the antigen are determined by ELISA as follows:

Microtiter plates are coated with the antigen (2 μg/ml in PBS) over night at 4°C. After washing three times with PBS-0.5%Tween™20, the wells are blocked with 2.5% non-fat dry milk in PBS for 1 hour at room temperature. Triplicate wells are incubated with serial sample dilutions for 1 h at room temperature. For IgG2a quantification, a standard serum is applied in serial dilutions to each plate. After washing, the wells are incubated with biotinylated mouse-anti rat IgG2a (serum) or IgA (mucus), diluted 1:1000 respectively for 1 h at room temperature. This is followed by 1h incubation at room temperature with avidin-alkaline phosphatase, diluted 1:500. The plates are developed by using alkaline phosphatase substrate (pNPP) and colour development is recorded at 405 nm after 20 minutes.

In vivo Trials and Results

A sample of rats was used in *in vivo* trials to determine the capacity for polymer particles according to Example 1 to act as a mucosal vaccine delivery system. Rats were immunised with either (i) 100μg HpaA + 25μg Cholera toxin(CT) (sample size: 4 rats); or (ii) the polymer particle delivery system of Example 1 (HpaA/PLG) containing 100μg HpaA (sample size: 6 rats). Three immunisations were carried out for each rat (one per week),

and sampling was carried out 7 weeks after the third immunisation. Negative controls were obtained by sampling prior to the first immunisation of the HpaA + Cholera toxin group.

The combination of protein (HpaA) + the mucosal adjuvant CT was used as a positive control.

The results are presented in Figures 5 and 6. It can be seen from the results in Fig. 6 that the polymer particles according to the present invention (HpaA/PLG in the figure) clearly deliver the antigen such that an anti-HpaA mucosal immune response is stimulated in the animals. This demonstrates the utility of the polymer particles according to the present invention as a delivery system for a vaccine, in the present trials a vaccine for treating and/or preventing *Helicobacter* infection. In particular, it is noteable that in figure 6 the result with the polymer particles according to the present invention is comparable to the result obtained with the positive control of HpaA + CT.

CLAIMS:

1. A method for producing polymer particles for use as a vaccine delivery system in which a water insoluble protein antigen is incorporated with particles comprising a polymer matrix, wherein the method comprises:-

(a) mixing an aqueous phase (W) with an organic phase(O) that is immiscible with W to produce a W/O emulsion, in which the water insoluble protein is solubilised in the W phase using a solubilising agent, and the O phase comprises the matrix polymer in an organic solvent;

(b) forming droplets of said W/O emulsion by dispersing the emulsion in a fluid medium, and removing said solvent from the O phase of the W/O emulsion droplets to thereby form polymer particles incorporating the water insoluble protein antigen; and

wherein in step (a) a stabilising agent is included in the W/O emulsion to promote the incorporation of the water insoluble protein with the polymer particles during step (b) by stabilising the W/O emulsion in the presence of said solubilising agent.

2. The method of claim 1, wherein more than one stabilising agent is included in the W/O emulsion.

3. The method of claim 1 or 2, wherein the or each stabilising agent is selected from polymers, polar lipids, and hydrophobic surfactants.

4. The method of any preceding claim, wherein a stabilising agent is used that is a polymer selected from poly(vinyl pyrrolidone), poly(vinyl alcohol), polysaccharides, polyethyleneoxide and water soluble proteins.

5. The method of any one of claims 1 to 3, wherein a stabilising agent is used that is a polar lipid selected from cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, glycolipids and phosphatidic acid.

6. The method of any one of claims 1 to 3, wherein a stabilising agent is used that is a non-ionic, hydrophobic surfactant selected from a sorbitan fatty acid ester, hydrophobic polyoxyethylene alkyl ether, sucrose ester, alkyl-glucopyranoside, polyglycerol polyricinoleate and block-copolymers of ethylene oxide with propyleneoxide and/or lactic acid.

7. The method of any one of claims 1 to 3, wherein a stabilising agent is used that is an anionic, hydrophobic surfactant selected from an alkylsulphate salt, dialkylsulphosuccinate salt, alkylbenzene sulphonate salt and a fatty acid salt.

8. The method of any one of claims 1 to 3, wherein a stabilising agent is used that is a cationic, hydrophobic surfactant selected from an alkyltrimethylammonium salt and a dialkyldimethylammonium salt.

9. The method of claim 2, wherein a sorbitan fatty acid ester is used as a stabilising agent.

10. The method of claim 2, wherein poly(vinyl pyrrolidone) and sodium 1,4-bis (2-ethylhexyl) sulphosuccinate are used as stabilising agents.

11. The method of any preceding claim, wherein more than one solubilising agent is used.

12. The method of any preceding claim, wherein a hydrophilic surfactant is used as a solubilising agent.

13. The method of claim 12, wherein the hydrophilic surfactant is a non-ionic surfactant selected from alkyl-glucopyranosides, alkyl-thioglucopyranosides, alkyl-maltosides, alkoyl-methyl glucamides, polyoxyethylene alcohols, polyoxyethylene alkyl phenols, emulphogens, polyoxyethylene sorbitol esters, polyoxyethylene fatty acid esters, hydrophilic polyoxyethylene alkyl ethers and digitonin.

14. The method of claim 12, wherein the hydrophilic surfactant is an anionic surfactant selected from cholates, alkylsulphonates, deoxycholates, alkylsulphates, oligooxyethylene dodecyl ether sulphates and sodium dodecylsarcosinate.

15. The method of claim 12, wherein the hydrophilic surfactant is a cationic surfactant selected from alkylpyridinium salts and alkyltrimethylammonium salts.

16. The method of claim 12, wherein the hydrophilic surfactant is a zwitterionic surfactant selected from CHAPS, CHAPSO, BIGCHAP, deoxy BIGCHAP, lyso phosphatidylcholine, alkylbetaines and sulphobetaines.

17. The method of any one of claims 1 to 11, wherein a chaotropic agent is used as a solubilising agent.

18. The method of claim 17, wherein the chaotropic agent is selected from a perchlorate, thiocyanate, guanidine, chlorate, iodide, bromide, nitrate and urea.

19. The method of any preceding claim, wherein the method is a Double Emulsion (W/O/X) Solvent Evaporation Technique for producing polymer particles for use as a vaccine delivery system, in which in step (b) the stabilised W/O emulsion is dispersed in a liquid phase (X) which is immiscible with the O phase to produce a W/O/X double emulsion comprising W/O droplets from which the solvent is evaporated, thereby producing said polymer particles incorporating the water insoluble protein antigen.

20. The method of any one of claims 1 to 18, wherein the method is a Double Emulsion (W/O/X) Solvent Extraction Technique for producing polymer particles for use as a vaccine delivery system, in which in step (b) the stabilised W/O emulsion is dispersed in a liquid phase (X) which is immiscible with the O phase to produce a W/O/X double emulsion comprising W/O droplets, wherein the X phase extracts said solvent from the O

phase of the droplets, thereby producing said polymer particles incorporating the water insoluble protein antigen.

21. The technique of claim 19 or 20, wherein a stabilising agent is included in the X phase.

22. The technique of claim 21, wherein a stabilising agent as defined in any one of claims 3 to 8 is used in the X phase.

23. The method of any one of claims 1 to 18, wherein the method is a spray drying technique for producing polymer particles for use as a vaccine delivery system, in which in step (b) the stabilised W/O emulsion is dispersed in a gaseous medium to form a spray of W/O emulsion droplets from which said solvent evaporates, thereby producing said polymer particles incorporating the water insoluble protein antigen.

24. The method of any one of claims 1 to 18, wherein in step (b) a fluid gas technique is used to form the polymer particles.

25. The method of claim 24, wherein the fluid gas technique is selected from GAS, SEDS, PCA, SAS and ASES.

26. The method of any preceding claim, wherein the protein antigen is a *Helicobacter* protein or fragment thereof.

27. The method of claim 26, wherein the protein antigen is a *Helicobacter pylori* protein or fragment thereof.

28. The method of claim 26 or 27, wherein said *Helicobacter* protein is a protein expressed on the surface of *Helicobacter*.

29. The method of claim 28, wherein the *Helicobacter* protein is a lipidated form of HpaA.

30. The method of claim 29, wherein the protein is a fully lipidated form of HpaA.

31. The method of claim 28, wherein the protein part of the lipidated HpaA protein has an amino acid sequence that is identical to, or substantially similar to, positions 28 to 260 of SEQ ID NO. 2 or 4.

32. The method of any preceding claim, wherein the matrix polymer is a homo- or co-polymer selected from one or more of polyesters, polyanhydrides, polyorthoesters, polycarbonates, polyamides, poly(amino acids), polyacetals, polycyanoacrylates, polyacrylates, biodegradable polyurethanes, non-erodable polyurethanes, polymers of ethylene-vinyl acetate, acyl substituted cellulose acetates, polysaccharides, polystyrenes, polyvinyl chloride, polyvinyl fluoride, poly(vinyl imidazole), chlorosulphonated polyolefins, polyethylene oxide, polyethers and polyoxalates.

33. The method of claim 32, wherein the polymer is a polyester homopolymer selected from polylactic acid, polyglycolic acid, polyhydroxybutyrate, poly(alpha hydroxyacids) and polycaprolactone.

34. The method of claim 32, wherein the polymer is a polyester co-polymer selected from poly(lactide-co-glycolide), poly(lactic-co-glycolic acid), poly(hydroxybutyrate-hydroxyvalerate) and poly(lactide-co-caprolactone).

35. The method of claim 34, wherein the matrix polymer is poly(D,L-lactide-co-glycolide).

36. The method of any preceding claim, wherein in step (a) the W phase is mixed with the O phase in a ratio by volume of 1:100 to 1:1.

37. A polymer particle vaccine delivery system obtainable by the method of any preceding claim.

38. A polymer particle vaccine delivery system in which a water insoluble protein antigen is incorporated with particles comprising a polymer matrix.

39. The vaccine delivery system of claim 38, wherein the protein antigen is a *Helicobacter* protein or fragment thereof.

40. The vaccine delivery system of claim 39, wherein the protein antigen is a *Helicobacter pylori* protein or fragment thereof.

41. The vaccine delivery system of claim 39 or 40, wherein said *Helicobacter* protein is a protein expressed on the surface of *Helicobacter*.

42. The vaccine delivery system of claim 41, wherein the *Helicobacter* protein is a lipidated form of HpaA.

43. The vaccine delivery system of claim 42, wherein the protein is a fully lipidated form of HpaA.

44. The vaccine delivery system of claim 42, wherein the protein part of the lipidated HpaA protein has an amino acid sequence that is identical to, or substantially similar to, positions 28 to 260 of SEQ ID NO. 2 or 4.

45. The vaccine delivery system of any one of claims 38 to 44, wherein the matrix polymer is a homo- or co-polymer selected from one or more of polyesters, polyanhydrides, polyorthoesters, polycarbonates, polyamides, poly(amino acids), polyacetals, polycyanoacrylates, polyacrylates, biodegradable polyurethanes, non-erodable polyurethanes, polymers of ethylene-vinyl acetate, acyl substituted cellulose acetates, polysaccharides, polystyrenes, polyvinyl chloride, polyvinyl fluoride, poly(vinyl imidazole), chlorosulphonated polyolefins, polyethylene oxide, polyethers and polyoxalates.

46. The vaccine delivery system of claim 45, wherein the polymer is a polyester homopolymer selected from polylactic acid, polyglycolic acid, polyhydroxybutyrate, poly(alpha hydroxyacids) and polycaprolactone.

47. The vaccine delivery system of claim 45, wherein the matrix polymer is a polyester co-polymer selected from poly(lactide-co-glycolide), poly(lactic-co-glycolic acid), poly(hydroxybutyrate-hydroxyvalerate) and poly(lactide-co-caprolactone).

48. The vaccine delivery system of claim 47, wherein the matrix polymer is poly(D,L-lactide-co-glycolide).

49. The vaccine delivery system of any one of claims 37 to 48, wherein the polymer particles have an average diameter of 0.05-20μm according to the volume size distribution.

50. A vaccine composition comprising the vaccine delivery system of any one of claims 37 to 49.

51. Use of the delivery system of any one of claims 37 to 49 in the manufacture of a vaccine composition, for the treatment of *Helicobacter* infection in a mammalian host.

52. Use of the delivery system of any one of claims 37 to 49 in the manufacture of a vaccine composition, for preventing or reducing the risk of *Helicobacter* infection in a mammalian host.

ABSTRACT

VACCINE DELIVERY SYSTEM AND METHOD OF PRODUCTION

The present invention concerns polymer particle vaccine delivery systems in which a water insoluble protein antigen, eg a lipidated HpaA protein, is incorporated with particles comprising a polymer matrix. The present invention also concerns a method for incorporating such a water insoluble protein antigen with a polymer matrix in order to produce a polymer particle vaccine delivery system. In addition, the invention also provides a vaccine composition comprising the polymer particle delivery system. The vaccine can be used to treat and/or reduce the risk of for example *Helicobacter* infection.

SEQUENCE LISTING

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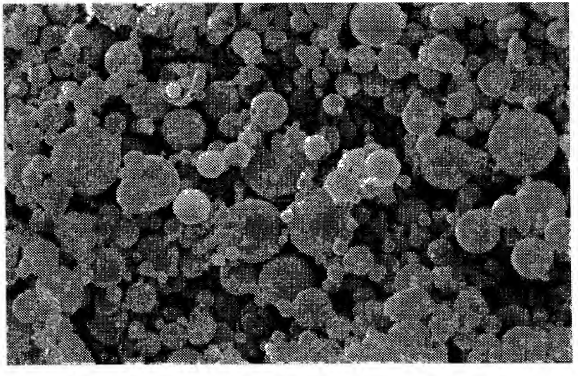

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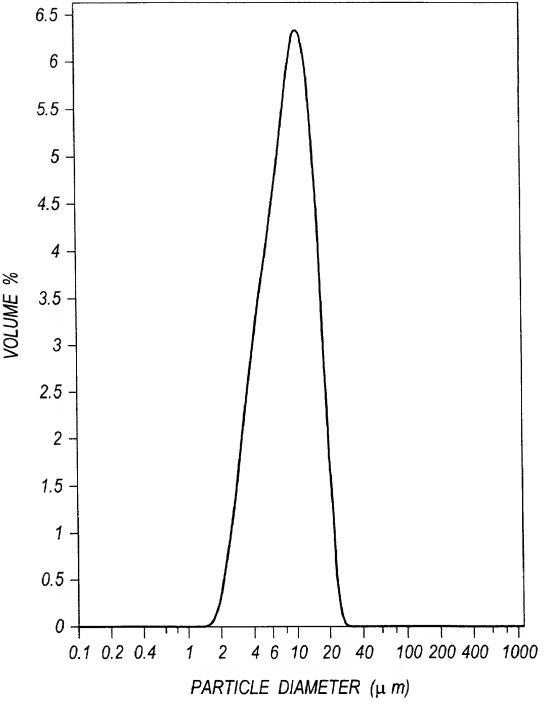
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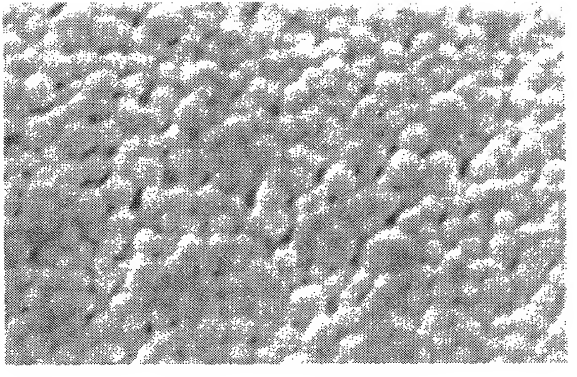
PARTICLE DIAMETER (μ m)

Fig.2



15kv X 20,000 1 μm

Fig.3

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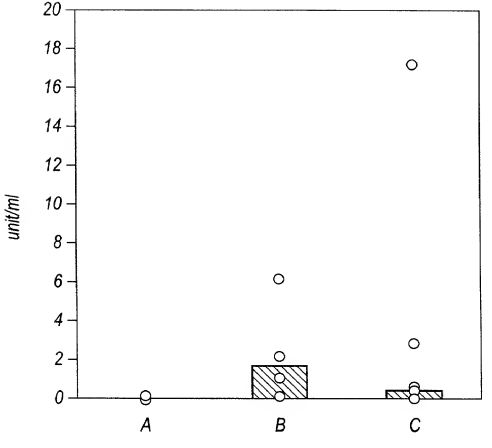
PARTICLE DIAMETER (μ m)

Fig.4



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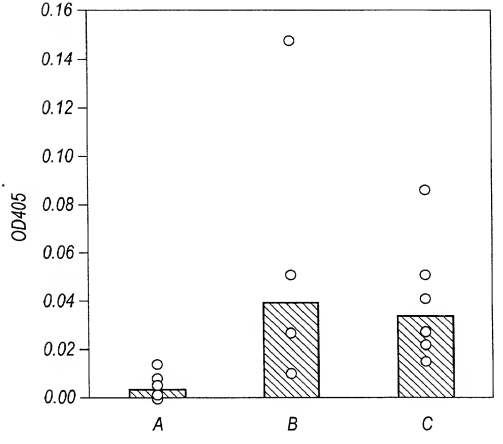

Fig.6

Docket Number (Optional) H 1939-1P US

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled VACCINE DELIVERY SYSTEM AND METHOD OF PRODUCTION the specification of which is attached hereto unless the following box is checked:

☒ was filed on 09 April 1999 as United States Application Number or PCT International Application Number SE99/00582 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

			Priority Not Claimed
9801288-3	Sweden	14 April 1998	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country	(Day(Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application Number)	(Filing Date)	(Status -- patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status -- patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

13

Edward V. Filardi, Reg. No. 25,757; Nels T. Lippert, Reg No. 25,888; Dimitrios Drivas, Reg. No. 32,218; Robert B. Smith, Reg. No. 28,538; Cecilia O'Brien Lofters, Reg. No. 33,434; David Bender, Reg. No. 35,445; John M. Genova, Reg. No. 32,224; Richard J. Sterner, Reg. No. 35,372; Hans-Peter G. Hoffmann, Reg. No. 37,352; Thelma C. Cleland, Reg.No. 40,948; Leslie Morioka, Reg. No. 40,304; John Scheibeler, Reg. No. 35,346; and Roy Waldron III, Reg. No. 42,208, all of the firm of WHITE & CASE Limited Liability Partnership, with offices at 1155 Avenue of the Americas, New York, New York 10036.

Address all telephone calls to _____ at telephone number (212) 819-8200

Address all correspondence to WHITE & CASE LLP
Patent Department
1155 Avenue of the Americas
New York, NY 10036-2787

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor
(given name, family name) Hans Carlsson

First inventor's signature Hans Carlsson Date: 26 April 1999

Residence Address Mölndal, Sweden SE Citizenship Swedish

Post Office Address Astra Hässle AB, S-431 83 Mölndal, Sweden

Full name of second joint inventor, if any
(given name, family name) Anette Larsson

Second inventor's signature _____ Date 26 April 1999

Residence Address Olofstorp, Sweden SE Citizenship Swedish

Post Office Address Astra Hässle AB, S-431 83 Mölndal, Sweden

Full name of third joint inventor, if any
(given name, family name) Erik Söderlind

Third inventor's signature _____ Date 26 April 1999

Residence Address Mölndal, Sweden SE Citizenship Swedish

Post Office Address Astra Hässle AB, S-431 83 Mölndal, Sweden